NOTE: This Statement of Work shall not be cited, quoted, nor distributed to any Testing Facility participating in the In Vitro Validation Study. Confidentiality must be maintained to ensure that test chemicals remain unknown to the Testing Facilities.

STATEMENT OF WORK

Procedures for Acquisition, Preparation, Solubility Testing, and Distribution of Test Chemicals for a Validation Study for *In Vitro* Basal Cytotoxicity Testing

April 26, 2002
Revision 1: May 8, 2002
Revision 2: June 21, 2002
Revision 3: September 17, 2002
Revision 4: October 11, 2002
Prepared by

The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)

National Institute of Environmental Health Sciences (NIEHS)
National Institutes of Health (NIH)
U.S. Public Health Service
Department of Health and Human Services

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NOTE: Revisions in this document are identified by footnotes, strike-out text (i.e., ~~deleted~~), and added verbiage (i.e., *italicized text*).

[3] Revised 9/17/02

STATEMENT OF WORK

Procedures for Acquisition, Preparation, Solubility Testing, and Distribution of Test Chemicals for a Validation Study for *In Vitro* Basal Cytotoxicity Testing

1.0 PROJECT OBJECTIVES AND GENERAL REQUIREMENTS

1.1 Project Objectives

This Statement of Work outlines and supports the procedures that the Contractor will initiate for the acquisition, preparation, solubility testing, and distribution of the test chemicals needed to perform two *in vitro* basal cytotoxicity assays (the BALB/c 3T3 Neutral Red Uptake [NRU] assay and the Normal Human Keratinocyte [NHK] Neutral Red Uptake [NRU] assay) for a multi-laboratory Validation Study. These assays, recommended in *Guidance Document On Using In Vitro Data To Estimate In Vivo Starting Doses For Acute Toxicity* (ICCVAM, 2001), use mammalian cell culture techniques to assess the basal cytotoxicity of chemicals.

A primary goal of this Validation Study is to evaluate the usefulness of the BALB/c 3T3 Neutral Red Uptake (NRU) and the Normal Human Keratinocyte (NHK) NRU assays for reducing and refining animal use for acute oral toxicity determinations of chemicals by predicting starting doses for *in vivo* rodent acute lethality assays.

The proposed Validation Study will determine IC_{20}, IC_{50}, and IC_{80} values for a test set of 72 chemicals with varying degrees of toxicity. This set of chemicals was selected separate and prior to this Statement of Work by the Study Management Team. The basis for selection of this test set is discussed in the Study Design document prepared by the Study Management Team.

The Contractor shall perform the following activities:

- Acquire 73 high quality and high purity (99% or greater when economically feasible) chemicals from reputable commercial sources
- Perform solubility tests on all chemicals using solvents and procedures that have been recommended to the test laboratories
- Repackage chemicals into multiple smaller units
- Code chemicals with a unique identification number so that chemicals can be provided to testing laboratories in a blinded fashion
- Distribute chemicals and health and safety information to the Testing Facilities
- Provide draft and final reports of these activities.

1.2 Response to the Statement of Work

Proposals submitted in response to this Statement of Work shall include:

a) A Work Plan
b) A timetable for project milestones
c) A cost estimate based on chemical acquisition, performance of solubility tests for all test chemicals, chemical coding, repackaging, and distribution to two U. S labs and one U. K. lab.

1.2.1 *General Capabilities*

The Contractor shall be capable of performing the following:

a) Prepare/provide Standard Operating Procedures (SOPs) for the performance of the activities outlined in **Section 1.1** (see **Section 1.4** – Definitions - SOPs)

b) Perform all aspects of the Test Chemical Preparation in accordance with Good Laboratory Practices (GLP).
c) Adhere to this Statement of Work throughout the Validation Study.

1.3 Guidelines

The Project Officer and/or her/his representatives (e.g., Study Management Team) may inspect and audit the Contractor to ensure that the Project Officer's minimum requirements and guidelines are being followed.

1.4 Definitions

Blinded/Coded Chemicals: Test chemicals supplied to the Testing Facilities that are coded and distributed by the Contractor such that only the Project Officer, Management Team, and the Contractor have knowledge of the contents of each test chemical vessel. The test chemicals will be purchased, aliquoted, coded, and distributed by the Contractor under the guidance of the NIEHS/NTP Project Officer and the Management Team.

Contractor: Facility that will initiate the acquisition, preparation, solubility testing, and distribution of the test chemicals needed to perform two *in vitro* basal cytotoxicity assays for a multi-laboratory *in vitro* Validation Study.

Good Laboratory Practices (GLPs): Regulations governing the conduct, procedures, and operations of toxicology laboratories; regulations to assure the quality and integrity of the data and to address such matters as organization and personnel, facilities, equipment, facility operations, test chemicals, and study protocol (Statement of Work) and conduct (U.S. Food and Drug Administration, Title 21 CFR Part 58; Environmental Protection Agency, Title 40 CFR Part 160).

Standard Operating Procedures (SOPs): Written documents that describe, in great detail, the routine procedures to be followed for a specific operation, analysis, or action; consistent use of an approved SOP ensures conformance with organizational practices, reduced work effort, reduction in error occurrences, and improved data comparability, credibility, and defensibility; SOPs also serve as resources for training and for ready reference and documentation of proper procedures;

Statement of Work: A description of test chemical preparation required for the *in vitro* Validation Study; defines all phases of the Validation Study and the purpose of the procedures; provides the details of test chemical acquisition, preparation, solubility testing, and distribution; provides guidance for the preparation of reports

Testing Facility: A laboratory that has been designated to participate in the *In Vitro* Validation Study; facilities identified in **Section 2.2.4**.

2.0 ORGANIZATION

2.1 Validation Study Sponsors

- National Institute of Environmental Health Sciences (NIEHS)
- The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)
- U.S. Environmental Protection Agency (U.S. EPA)
- The European Centre for the Validation of Alternative Methods (ECVAM).

2.2 Management Team

2.2.1 *Project Management and Chemical Distribution Team*

Ms. Molly Vallant (NIEHS) – NIEHS Project Officer for BioReliance, Inc.
NIEHS
MD E1-03
P.O. BOX 12233
RTP, NC 27709

Dr. Martin L. Wenk (BioReliance, Inc.) – Chemical acquisition, preparation, solubility testing, and distribution
BioReliance Corporation
14920 Broschart Road
Rockville, Maryland 20850-3349

2.2.2 *Contract Management*

Ms. Jackie Osgood (NIEHS) – Contracting Officer
Mr. Don Gula (NIEHS) – Contracting Officer

2.2.3 *Study Management Team*

2.2.3.1 ***NIEHS/NICEATM***

Dr. William S. Stokes (NICEATM/NIEHS) – Co-chair – Study Management Team
Dr. Judy Strickland (NICEATM/ILS) – Project Coordinator
Mr. Michael Paris (NICEATM/ILS) – Assistant Project Coordinator
Dr. Ray Tice (NICEATM/ILS) – Technical Advisor

NICEATM
79 T.W. Alexander Drive
Bldg. 4401, MD-EC-17
3rd Floor, Room 3126
P.O. Box 12233
Research Triangle Park, NC 27709

2.2.3.2 ***ECVAM***

Professor Michael Balls – Co-chair – Study Management Team
Dr. Silvia Casati
Dr. Andrew Worth

European Commission
Joint Research Centre
Institute for Health and Consumer Protection
Management Support Unit - TP 202
I-21020 Ispra (VA) - Italy

2.2.4 *Testing Facilities*

XXX, Safety Officer
Institute for *In Vitro* Sciences (IIVS)
21 Firstfield Road
Suite 220
Gaithersburg, MD 20878

Bill Cappuccio, Safety Officer
5183 Blackhawk Rd
E3330/Room 278
Aberdeen Proving Ground-EA, MD 21010
410-436-7462

Rodger Dainty, Safety Officer
School of Biomedical Sciences
University of Nottingham Medical School
Queen's Medical Centre
Nottingham, NG7 2UH UK

3.0 CONTRACTOR AND KEY PERSONNEL

3.1 Contractor

The Contractor shall have competence in chemical acquisition, preparation, solubility testing, and distribution and shall provide competent personnel, adequate facilities, equipment, supplies, proper health and safety guidelines, and satisfactory quality assurance procedures.

3.1.1 *Personnel*

3.1.1.1 ***Facility Management***

The facility management is responsible for establishing scientific guidelines and procedures, training and supervision of professional and technical staff, and evaluation of results and performance within their discipline area relative to the Project Officer's stated requirements. The manager must maintain records of the qualifications, training and experience, and a job description for each professional and technical individual involved in test chemical acquisition, preparation, solubility testing, and distribution.

3.1.1.2 ***Study Director***

A scientist or other professional of appropriate education, training, and experience in chemical acquisition, preparation, solubility testing, and distribution, or combination thereof, shall be the Study Director. The Study Director has the overall responsibility for the technical conduct of chemical acquisition, preparation, solubility testing, and distribution for the Validation Study (e.g., GLP adherence) and shall be responsible for determining test acceptance. The Study Director shall be responsible for providing SOPs that incorporate pertinent information obtained from the Statement of Work. Other duties include the interpretation and analysis of test chemical solubility data, documentation of all study aspects (including maintenance of a Study Workbook), and production of all draft and final written reports.

3.1.1.3 ***Quality Assurance (QA) Director***

The Quality Assurance Director shall **monitor** all tasks and assure conformance with GLP requirements (i.e., facilities, equipment, personnel, methods, practices, records, controls, transference of data into software, SOPs). Quality Assurance Director or unit can be any person or organizational element, except the Study Director, designated by Contractor management to perform the duties relating to quality assurance of the studies and tasks. The Quality Assurance duties are not a substitute for the Study Director duties.

3.1.1.4 ***Scientific Advisor(s)***
Scientists or other professionals of appropriate education, training, and experience in chemical acquisition, preparation, solubility testing, and distribution who provide scientific guidance to the Study Director and other laboratory personnel.

3.1.1.5 ***Laboratory Technician(s)***
Each individual engaged in the conduct of or responsible for the supervision of a study shall have education, training, and experience, or combination thereof, to enable that individual to perform the assigned duties. The individuals must be trained in GLP requirements and technical ability must be documented as per GLP requirements.

3.1.1.6 ***Safety Officer***
The Contractor shall designate a Safety Officer who will provide a sealed health and safety information package that will accompany the test chemicals to the Test Facilities. A duplicate package will be provided to the Project Officer and Management Team.

3.1.2 ***Facilities, Equipment, and Supplies***

3.1.2.1 ***Laboratory***
The Contractor must provide a designated laboratory/area to ensure that test chemical preparation and solubility testing can be performed under clean conditions. Potential for cross-contamination of chemicals should be minimal.

3.1.2.2 ***Equipment***
The Contractor must provide at a minimum the following equipment:
a) Water bath (37^{o}C)
b) Sonication unit
c) Vortex unit
d) Pippettors (micropipettors,)
e) Computer (for data transformation and analysis)
f) Balance
g) pH meter

All equipment maintenance and calibration shall be routinely performed and documented as per GLP guidelines and Contractor procedures

3.1.2.3 ***Supplies***
All cell culture reagents must be labeled so as to indicate source, identity, concentration, stability, preparation and expiration dates, and storage conditions.
a) Dulbecco's Modification of Eagle's Medium (DMEM) without L-Glutamine; should have Hanks' salts and high glucose [4.5gm/l] (e.g., ICN-Flow Cat. No. 12-332-54)
b) L-Glutamine 200 mM (e.g., ICN-Flow # 16-801-49)
c) New Born Calf Serum (NBCS) (e.g., Biochrom # SO 125)
d) Dimethyl sulfoxide (DMSO), U.S.P. analytical grade. DMSO shall be stored under nitrogen at –20^{o}C.
e) Ethanol (ETOH), U.S.P. analytical grade (100%, non-denatured)

f) Keratinocyte Basal Medium without Ca^{++} (KBM®, Clonetics CC-3104) that is completed by adding the *KBM® SingleQuots®* ~~Bullet Kit®~~[2] (Clonetics CC-4131) to achieve the proper concentrations of epidermal growth factor, insulin, hydrocortisone, antimicrobial agents, bovine pituitary extract, and calcium (e.g., Clonetics Calcium SingleQuots®, CC-4202)*.

g) Penicillin/streptomycin solution (e.g. ICN-Flow # 16-700-49)

* BioWhittaker, 8830 Biggs Ford Road, Walkersville, MD 21793 (http://www.cambrex.com/subsidiaries/s%2Dbw%5Finc/s%2Dbiowhittaker%2Dinc%2Dcontact2.htm)

3.1.3 *Health and Safety*

The Contractor shall conform to all local, state, and federal statutes in effect at the time of this study.

3.1.4 *Quality Assurance*

The Contractor shall conduct the acquisition, preparation, solubility testing, and distribution of test chemicals in compliance with Good Laboratory Practice (GLP) Standards (U.S. Food and Drug Administration, Title 21 CFR Part 58; Environmental Protection Agency, Title 40 CFR Part 160). The appropriate QA unit (as per GLPs) shall audit the procedures and final report.

The Final Report shall be audited by the Quality Assurance unit of the Contractor for GLP compliance and a QA Statement shall be provided by the Contractor. The Final Report shall identify: 1) the phases and data inspected, 2) dates of inspection, and 3) dates findings were reported to the Study Director and Contractor management. The QA Statement shall identify whether the methods and results described in the Final Report accurately reflect the raw data produced during the study.

4.0 TEST PHASES AND SCHEDULE

4.1 Study Timeline

The following timeline is for the **laboratory testing aspect** of the *In Vitro* Validation Study. The Contractor shall provide the required chemicals in a timely fashion so that each phase of the study can start on the appointed date.

[2] Revised 6/21/02

TASK	WEEK	ESTIMATED DATE
Statement of Work issued by NIEHS to the Testing Facility	0	March 29, 2002
Response /Proposal received from the Testing Facility	6	May 10, 2002
~~Award of Contracts~~[2]	~~9~~[2]	~~May 29, 2002~~[2]
Submission of *Study Protocol, CVs of Key Personnel,* SOPs[2]	11	June 12, 2002
Award of Contracts[2]	*13*[2]	*June 28, 2002*[2]
Start Testing – Phase I (Phase Ia)	~~14~~*18*[2]	July ~~1~~*29*[2], 2002
End Phase Ia	~~18~~*22*[2]	~~July~~ *August* 2~~6~~9[2], 2002
Begin Phase Ib	~~22~~*26*[2]	~~August~~ *September* ~~29~~*26*[2], 2002
End Phase Ib	~~27~~*31*[2]	October ~~1~~*29*[2], 2002
Begin Phase II	~~31~~*36*[2]	~~October~~ *December* 2~~9~~[2], 2002
End Phase II	~~42~~*46*[2]	~~January~~ *February* ~~13~~*10*[2], 2003
Begin Phase III	~~48~~*52*[2]	~~February~~ *March*[2] 26, 2003
Final Report (Phase III) to SMT	~~85~~*89*[2]	~~November~~ *December* ~~11~~*9*[2], 2003

4.2 Deliverables

The following schedule of deliverables is for **the acquisition, preparation, solubility testing and distribution of test chemicals**.

	ESTIMATED DUE DATES (to Project Officer)			
Submission of SOPs for **Section 1.1** activities	**Week 11**		**June 12, 2002**	
REPORTS	**PHASE Ia**	**PHASE Ib**	**PHASE II**	**PHASE III**
Biweekly Reports	a	a	a	a
Draft Phase Reports	Week ~~13~~*17* ~~June~~ *July* 2~~4~~6[2], 2002 [b]		Week ~~29~~*33* ~~Oct~~*Nov*. ~~1~~6*13*[2], 2002 [b]	Week ~~44~~*48* ~~Jan~~*Feb*. ~~29~~*26*[2], 2003 [b]
Draft Final Report (all phases combined)	Week ~~48~~*52* *March*~~Feb.~~[2] 26, 2003 [c]			
Final Report (all phases combined)	Week ~~50~~*54* ~~March~~ *April* 9~~12~~[2], 2003 [d]			

a Biweekly reports shall begin at the time of implementation of the contracts and continue until the final report is submitted.

b Draft Phase Reports shall be submitted to the Project Officer no later than the dates provided (at least two weeks before shipment of chemicals to the Test Facilities).

c Draft Final Report shall be submitted to the Project Officer no later than the date provided (at the most one month after final shipment of chemicals to the Test Facilities).

d Final Report shall be submitted to the Project Officer no later than the date provided (at the most one month after the Project Officer receives the Draft Final Report.

[2] Revised 6/21/02

The following schedule is for the **distribution of test chemicals** to the Testing Facilities.

	ESTIMATED DUE DATES (to Testing Facilities)			
CHEMICAL SHIPPING TO TESTING FACILITIES[a]	**PHASE Ia**	**PHASE Ib**	**PHASE II**	**PHASE III**
Positive Control (SLS)	Before July ~~1~~*29*[2], 2002	---	---	---
Phase Ib (3 chemicals)	---	Before ~~August~~ *September* ~~29~~*26*[2], 2002	---	---
Phase II (9 chemicals)	---	---	Before ~~October~~ *December* 2~~9~~[2], 2002	---
Phase III (60 chemicals)	---	---	---	Before ~~February~~ *March*[1] 26, 2003

a Dates for chemical shipments are to ensure that the Testing Facilities receive Test Chemicals prior to the start dates of each lab testing phase. Phase III chemicals shall be shipped as one group of 60 chemicals. Chemicals for each phase are identified in Addendum IV.

4.3 *In Vitro* Validation Study Phases

Phase I: The training phase for laboratory personnel. This phase includes developing a positive control database (Phase Ia) and testing three unknown chemicals (Phase Ib).
Phase II: The qualification phase. This phase requires testing nine blinded/coded chemicals in the same *in vitro* cytotoxicity assays and in the same concentration-response fashion as in Phase Ib.
Phase III: Testing 60 blinded/coded chemicals in the same manner as in Phases I and II.

4.4 Report Submission Timelines

4.4.1 *Draft Reports*

Draft reports for each phase shall be submitted to the Project Officer as per **Section 4.2**.

4.4.2 *Final Report*

The Final report shall be submitted to the Project Officer as per **Section 4.2**.

5.0 ACQUISITION, PREPARATION, AND DISTRIBUTION OF TEST CHEMICALS

5.1 Test Chemicals

5.1.1 *Range of Toxicities*

The chemicals proposed for the Validation Study are representative of a range of toxicities and are relevant with regard to human exposure potential. The test chemicals

[2] Revised 6/21/02

will represent each of the Globally Harmonized System (GHS) classification groups for rat oral LD50s: ≤ 5 mg/kg, >5 ≤ 50 mg/kg, >50 ≤ 300 mg/kg, >300 ≤ 2000 mg/kg, >2000 ≤ 5000 mg/kg, and >5000 mg/kg (OECD, 2001). Addenda III and IV provide the list of test chemicals for the *In Vitro* Validation Study.

5.1.2 ***Procurement of Test Chemicals***
The Contractor shall purchase 73 chemicals specified in Addenda III and IV (72 "test chemicals" and one "positive control") from commercial manufacturers. Chemical purity shall be 99% or greater when economically feasible. Chemical information from the manufacturers shall be collected as specified in **Section 7.1.2** and reported as indicated in Addendum I. Chemicals shall be stored as recommended by the manufacturer.

5.1.3 ***Dispensing Chemicals***
While preparing the purchased chemicals for distribution to the Testing Facilities, only one bulk substance shall be dispensed at any time. All test samples shall be sealed and labeled before dispensing the next substance. Once test samples have been dispensed into aliquots, they shall be returned to appropriate storage conditions until they are dispatched.

During dispensing, all test chemicals, with the exception of the positive control, will be randomly blinded/coded so that testing by the Testing Facilities will be conducted on chemicals with a masked identity. Each chemical shall have a code that is unique for each Testing Facility (i.e., no chemical shall have the same code in any Testing Facility). The Contractor shall dispense 4 g of test chemical/Testing Facility (see Addendum V for assumptions used to determine the amount of chemical/Testing Facility) into clean, sterile containers, and assign unique code identifiers, and archive two additional samples. About 100 g of the positive control shall be distributed to each lab and one additional sample shall be archived.

5.1.4 ***Shipment of Chemicals***
After dispensing and labeling chemical aliquots with unique codes, the Contractor shall ship a set of the test chemicals, including the positive control, to the each of three Testing Facilities. Two Facilities will be in the US and one will be in the United Kingdom. The Contractor will package test chemicals so as to minimize damage during transit and will ship them to each Testing Facility according to proper regulatory procedures. Except for the positive control in Phase Ia, chemicals are to be packaged and shipped so as to conceal their identities. Test chemicals shall be shipped under conditions that will preserve the integrity of the chemicals. The Contractor shall notify the Testing Facilities (and the Project Officer) when the test chemicals are shipped so as to prepare for receipt.

The Contractor will retain the archived chemicals, which may be required for retesting or purity analysis, until the completion of the Validation Study.

5.1.4.1 ***Distribution Phases***
Phase Ia: For Phase I, the positive control chemical identified in Addendum III shall be distributed to all three Testing Facilities.
Phase Ia: For Phase Ib, the three (3) blinded/coded chemicals identified in Addendum III shall be distributed to all three Testing Facilities.
Phase II: Nine (9) blinded/coded chemicals identified in Addendum III shall be distributed to all three Testing Facilities.

Phase III: Sixty (60) blinded/coded chemicals identified in Addendum III shall be distributed to the Test Facilities. Chemicals will be shipped –as a group of 60 chemicals.

5.1.5 ***Receipt of Chemicals by the Testing Facilities***

With the exception of the positive control shipment, which shall be shipped directly to the Study Director, the chemical shipments shall be addressed to the Testing Facility Safety Officers and accompanied by a sealed information packet containing the appropriate health and safety procedures for use (i.e., Material Safety Data Sheets (MSDS) or equivalent documentation with proper protection, procedures for accidental ingestion or contact with skin or eyes, and procedures for containing and recovering spills) and a disclosure key for identifying test chemicals by code. The shipment shall include instructions for the Testing Facility Safety Officer to:

1) Immediately notify the Contractor and Study Project Coordinator upon receipt of chemicals,
2) Retain the health and safety package and pass the test chemicals to the Study Director without revealing the identities of the test chemicals,
3) Notify the Management Team if Test Facility personnel open the health and safety packet at any time during the Validation Study, and
4) Return the unopened health and safety package to the Contractor after testing is complete. The Contractor shall immediately notify the Project Officer regarding chemical receipt.

If regulatory transportation requirements dictate that each package must display a list of the chemicals it contains on the outside of the package, the Contractor shall direct the Testing Facility Safety Officer to remove it prior to passing the chemicals to the Study Director.

5.1.6 ***Test Chemical Information for the Study Director***

The Contractor shall supply, with each test chemical, data sheets giving a minimum of essential information, including color, odor, physical state, weight or volume of sample, specific density for liquid test chemicals, and storage instructions. The Study Director shall receive this information from the Safety Officer.

5.2 Handling of Test Chemicals

Appropriate routine safety procedures shall be followed in handling the test chemicals. The Contractor shall include instructions to the Test Facilities to treat all blinded/coded test chemicals as ***very hazardous and potentially carcinogenic.*** After the studies are completed, the remaining test chemicals will be returned by the Testing Facilities to the Contractor.

5.3 Determination of Purity, Composition, and Stability of Test Chemicals

As indicated in **Section 7.1.2**, the Contractor will be directly responsible for collecting information (from manufacturer and supplier documentation) on the analytical purity, composition, and stability of the test chemicals and the positive control material, and their homogeneity (via Contractor solubility studies) in the vehicle.

6.0 SOLUBILITY DETERMINATION OF TEST CHEMICALS

The Contractor shall determine solubility of the test chemicals in the same manner as recommended to the Testing Facilities (i.e., by following the hierarchy below).

6.1 Cell Culture Media and Control Material

6.1.1 *Test Chemical Medium Solvents*

6.1.1.1 ~~*Treatment*~~ *Chemical Dilution*[3] *Medium (BALB/c 3T3 NRU)*

Serum-free[3] Dulbecco's Modification of Eagle's Medium (DMEM) [see **Section 3.1.2.3.a**] buffered with sodium bicarbonate and supplemented with (final concentrations in DMEM are quoted):

~~5% NBCS~~[3]
4 mM Glutamine
~~100~~ *200* IU/*mL*[3] Penicillin
~~100~~ *200* µg/ml[3] Streptomycin

This *serum-free*[3] medium is used in the assay for ~~application of~~*dissolving*[3] test chemicals *prior to application*[3] to the 3T3 cells.

6.1.1.2 *Routine Culture Medium (NHK NRU)*

KBM® (Clonetics CC-3104) supplemented with KBM® SingleQuots® (Clonetics CC-4131) and Clonetics Calcium SingleQuots® (CC-4202) to make 500ml of medium. Final concentration of supplements in medium are: ~~A modified MCDB 153 formulation such as Clonetics® Keratinocyte Basal Medium (KBM®) supplemented with (final concentrations in KBM® are quoted):~~[2]

0.*000*1 ng/ml[2] Human recombinant epidermal growth factor
5 *µ*g/ml[2] Insulin
0.5 g/ml[2] Hydrocortisone
~~50~~ *30 µ*g/ml[2] Gentamicin
~~50~~ *15* ng/ml[2] Amphotericin B
0.10 mM Calcium
~~2 ml 7.5 mg/ml~~*30 µg/ml*[2] Bovine pituitary extract.

This medium is used in the assay as the routine culture medium and for application of test chemicals to the NHK cells. *Complete media should be kept at 4°C and stored for no longer than two weeks.*[2]

NOTE: KBM® SingleQuots® contain the following stock concentrations and volumes:[2]

0.1 ng/ml	*hEGF*	*0.5 ml*[2]
5.0 mg/ml	*Insulin*	*0.5 ml*[2]
0.5 mg/ml	*Hydrocortisone*	*0.5 ml*[2]
30 mg/ml	*Gentamicin, 15 ug/ml Amphotericin-B*	*0.5 ml*[2]
7.5 mg/ml	*Bovine Pituitary Extract (BPE)*	*2.0 ml*[2]

Clonetics Calcium SingleQuots® are 2 ml of 300mM concentration of calcium.[2] *165 ul of solution per 500 ml calcium-free medium equals 0.10 mM calcium in the medium.*[2]

[3] Revised 9/17/02
[2] Revised 6/21/02

6.1.2 ***Positive Control (PC)***
Sodium Lauryl Sulfate ([SLS], CAS # 151-21-3) will be the positive control material for the *In Vitro* Validation Study.

6.2 Preparation of Test Chemical

All chemicals (including the positive control [SLS]) shall be weighed on a calibrated balance (including liquid test chemicals) and added to the appropriate solvent (**Section 6.2.1**). Test chemicals must be at room temperature before dissolving. Preparation under red light or yellow light may be necessary, if rapid photodegradation is likely to occur. The solutions must not be cloudy nor have noticeable precipitate.

6.2.1 ***Dissolving the Test Chemical***[3]
The hierarchy specified in **Sections 6.2.1.1 to 6.2.1.3** (i.e., culture medium, DMSO, ethanol) shall be followed for dissolving the test chemicals and positive control. Both assay-specific culture media specified in **Section 6.1.1** *(i.e., Chemical Dilution Medium for 3T3 cells and Routine Culture Medium for NHK cells)* must be tested.

Approximately 100 mg (100,000 μg) of the test chemical will be weighed into a glass tube and the weight will be documented. Assay-specific media will be added to the vessel so that the concentration is 200,000 μg/ml (200 mg/mL) (i.e., approximately 0.5 mL). The solution is mixed as specified in Section 6.2.1.1. If complete solubility is achieved, then additional solubility procedures are not needed. If only partial solubility is achieved, follow the test chemical dissolving steps in Table 1, derived from EPA (1998), to add additional medium in steps until the concentration is a minimum of 2,000 μg/mL (2 mg/mL). If complete solubility at 2,000 μg/mL in medium can't be attained, then repeat the solubility steps using the other solvent(s) in the solubility hierarchy. Test chemicals that are only soluble in DMSO or ethanol will be prepared at 500,000 μg/mL as the highest concentration of stock solution.

Table 1: Determination of Solubility in Media

STEP	***1***	***2***	***3***	***4***	***5***
Total Volume of Medium	*0.5 mL*	*2.5 mL*	*5.0 mL*	*2.0 mL*	*10.0 mL*
Concentration of Test Chemical (Add 100 mg to a tube. Add the first volume of medium. Dilute with subsequent volumes if necessary.)	*200,000 μg/mL (200 mg/mL)*	*40,000 μg/mL (40 mg/mL)*	*20,000 μg/mL (20 mg/mL)*		
Concentration of Test Chemical (Add 20 mg to a large tube. Add the first volume of medium. Dilute with subsequent volume if necessary.)				*10,000 μg/mL (10 mg/mL)*	*2,000 μg/mL (2.0 mg/mL)*
If test chemical is insoluble in medium at 2000 μg/mL, then attempt to dissolve chemical in DMSO. Actual volume of solution can be determined after test chemical is dissolved and solution is measured using a calibrated instrument (e.g., micropipettor, or serological pipette). The actual stock concentration can be calculated accordingly.					

Example: If complete solubility is not achieved in 0.5 mL medium (Step 1) using the mixing procedures specified in ***Section 6.2.1.1, b-d,*** *then 2.0 mL must be added to obtain a total volume of 2.5 mL (Step 2). Chemical and medium are again mixed as prescribed in* ***Section 6.2.1.1*** *in an attempt to dissolve. If solubility is not achieved at Step 2, then 2.5 mL medium is added in Step 3.*

[3] Section 6.2.1 replaced 9/17/02

*Chemical and medium are again mixed as prescribed in Section **6.2.1.1** in an attempt to dissolve. No additional weighing of the chemical is required until Step 4.*

6.2.1.1 Chemical Dilution Medium/Routine Culture Medium

a) *Dissolve test chemical in Chemical Dilution Medium and Routine Culture Medium as in Step 1 of **Table 1**.*
b) *Gently mix. Vortex for 1-2 minutes.*
c) *If test chemical hasn't dissolved, use sonication for up to five minutes.*
d) *If sonication doesn't work, then warm solution to 37°C.*
e) *Proceed to Step 2 (and Steps 3-5, if necessary) of **Table 1** and repeat procedures b-d.*

6.2.1.2 DMSO

*If the test chemical doesn't dissolve in the Chemical Dilution Medium or Routine Culture Medium, then follow the dilution steps in **Table 1A** and mixing steps a) through e) in **Section 6.2.1.1** using DMSO instead of Chemical Dilution Medium/Routine Culture Medium.*

6.2.1.3 Ethanol

*If the test chemical doesn't dissolve in DMSO, then follow the dilution steps in **Table 1A** and mixing steps a) through e) in **Section 6.2.1.1** using ethanol instead of DMSO.*

Table 1A: Determination of Solubility in DMSO and Ethanol

Steps	***1***	***2***	***3***	***4***	***5***	***6***
Total Volume of DMSO or Ethanol	*0.2 mL*	*0.5 mL*	*2.5 mL*	*5.0 mL*	*2.0 mL*	*10.0 mL*
Concentration of Test Chemical (Add 100 mg to a tube. Add the first volume of solvent. Dilute with subsequent volumes if necessary.)	*500,000 µg/mL (500 mg/mL)*	*200,000 µg/mL (200 mg/mL)*	*40,000 µg/mL (40 mg/mL)*	*20,000 µg/mL (20 mg/mL)*		
Concentration of Test Chemical (Add 20 mg to a tube. Add the first volume of solvent. . Dilute with subsequent volume if necessary.)					*10,000 µg/mL (10 mg/mL)*	*2,000 µg/mL (2.0 mg/mL)*
If test chemical is insoluble in DMSO at 2000 µg/mL, then attempt to dissolve chemical in ethanol. Actual volume of solution can be determined after test chemical is dissolved and solution is measured using a calibrated instrument (e.g., micropipettor, or serological pipette). The actual stock concentration can be calculated accordingly.						

*If the test chemical does not dissolve in Chemical Dilution Medium/Routine Culture Medium, DMSO, or ethanol, at 2 mg/mL, then repeat the entire solubility procedure with each solvent (in the order of Chemical Dilution Medium/Routine Culture Medium, DMSO, and ethanol) using the dilution steps in **Table 1B** and mixing steps a) through e) in **Section 6.2.1.1**.*[4]

[4] Added 10/11/02

Table 1B: Further Determination of Solubility in Chemical Dilution Medium/Routine Culture Medium, DMSO, or Ethanol[4]

STEP	*6*	*7*	*8*	*9*	*10*
Total Volume of Solvent	*5 mL*	*10 mL*	*20 mL*	*40 mL*	*100 mL*
Concentration of Test Chemical (Add 5 mg to a tube. Add the first volume of solvent. Dilute with subsequent volumes if necessary.)	*1,000 µg/mL (1 mg/mL)*	*500 µg/mL (0.5 mg/mL)*	*250 µg/mL (0.25 mg/mL)*	*125 µg/mL (0.125 mg/mL)*	*50 µg/mL (0.05 mg/mL)*
If test chemical is insoluble in medium at 50 µg/mL, then attempt to dissolve chemical in DMSO and then ethanol. Actual volume of solution can be determined after test chemical is dissolved and solution is measured using a calibrated instrument. The concentration can be calculated accordingly.					

~~Approximately 100 *200* mg (100*200*,000 µg)[2] of the test chemical will be weighed into a glass tube and the weight will be documented. Assay-specific culture media will be added to the vessel so that the concentration is 1*2*,000,000 µg/ml (1000 *2000* mg/ml)[2] (i.e., approximately 0.1 ml). If complete solubility is achieved, then additional solubility procedures are not needed. If only partial solubility is achieved, follow the test chemical dissolving steps in Table 1, derived from EPA (1998), to add additional medium in steps until the concentration is a minimum of 100*200*,000 µg/ml (100 *200* mg/ml)[2]. If complete solubility at 100,000 µg/ml in culture medium can't be attained, then repeat the solubility steps using the other solvent(s) in the solubility hierarchy. Test chemicals that are only soluble in DMSO or ethanol will be prepared at 1*2*,000,000 µg/ml[2] as the highest concentration of stock solution.~~

~~Table 1: Determination of Solubility~~

~~**Solubility Data**~~	~~**Step 1**~~	~~**Step 2**~~	~~**Step 3**~~
~~Total volume of medium added (ml)~~	~~0.1~~	~~0.5~~	~~1.0~~
~~Total volume of DMSO or ethanol added (ml)~~	~~0.1~~	~~****0.5*~~[2]	~~****1.0*~~[2]
~~Approximate solubility (µg/ml)~~	~~≥ 1*2*,000,000~~[2]	~~200*400*,000~~[2]	~~100*200*,000~~[2]

~~**6.2.1.1 *Treatment Medium/Routine Culture Medium)***~~

~~*a)f)* Dissolve test chemical in Treatment Medium and Routine Culture Medium~~
~~*b)g)* Gently mix. Vortex for 5-10 seconds*1-2 minutes*.[2]~~
~~*c)h)* If test chemical hasn't dissolved, use sonication (up to five minutes).~~
~~*d)i)* If sonication doesn't work, then warm solution to 37°C.~~

~~**6.2.1.2 *DMSO***~~

~~If the test chemical doesn't dissolve in the Treatment Medium/Routine Culture Medium, then follow steps a) through d) in **Section 6.2.1.1** using DMSO instead of Treatment Medium/Routine Culture Medium.~~

6.2.1.3 *Ethanol*

~~If the test chemical doesn't dissolve in DMSO, then follow steps a) through d) in **Section 6.2.1.1** using ethanol instead of DMSO.~~

[2] Revised 6/21/02
[2] Revised 6/21/02

6.2.2 *pH of Solutions*

Measure the pH (using pH paper) of the highest concentration of test chemical dissolved in the culture media. Document the pH and note the color of each test chemical concentration in medium.

7.0 DATA COLLECTION

7.1 Nature of Data to be Collected

7.1.1 *Solubility Studies*

The Contractor shall record all information pertinent to the solubility of the test chemical:

a) *Approximate t*~~T~~[3]est chemical solubility in *all solvents tested (i.e., media, DMSO, and/or ethanol) in weight per unit volume (i.e. mg/mL) estimated by following the step-wise solubility protocol* ~~culture medium at a minimum of 100~~*200*,000[2] ~~µg/ml~~[3]
b) pH of test chemical in culture medium; color of culture medium
~~c) Test chemical solubility in DMSO or ethanol at 1~~*2*,000,000[2] ~~µg/ml~~[3]
d) Need of vortexing, sonication, and/or heating

The Contractor shall provide this information to the Study Management Team via the Project Officer by the avenues described in Section 8. **This information shall NOT be provided to the Testing Facilities.** Information to be provided to the Testing Facilities is specified in Sections 5.1.5 and 5.1.6.

7.1.2 *Chemical Information*

The Contractor shall supply at a minimum the following information about each test chemical and report as specified in Addendum I.

a) Purity
b) CAS #
c) Supplier
d) Specification sheets
e) Certificates of analysis
f) Material Safety Data Sheet (MSDS)
g) Color
h) Odor
i) Physical state
j) Weight or volume of sample distributed to the Testing Facility
k) Specific density for liquid test chemicals
l) Storage instructions
m) Chemical hazards
n) Special handling instructions
o) Amount of material archived

[Note: Much of the information will be in the MSDS.]

7.2 Type of Media Used for Data Storage

Originals of the raw data (the Study Workbook) and copies of other raw data such as instrument logs shall be collected and archived at the end of the study (under the direction of the Study Director), according to GLP-compliant procedures. Data that are stored electronically shall be periodically copied, and backup files shall be produced and maintained.

[2] Revised 6/21/02
[3] Revised 9/17/02

7.3 Documentation

Original raw data that shall be collected shall include but are not limited to the following:

- Data recorded in the Study Workbook, which shall consist of all recordings of all activities related to acquisition, preparation, solubility testing, and distribution of the test chemicals;
- Other data collected as part of GLP compliance
  - Equipment logs
  - Equipment calibration records

8.0 DRAFT AND FINAL REPORTS

Biweekly Reports: The Contractor will provide a biweekly progress report to the Project Officer and copied to the Project Coordinators of the Study Management Team (See **Section 4.2** and Addendum I). These reports will include raw and interim data as the study progresses. These reports will be in electronic format (i.e., email with Microsoft® Word (or equivalent) or Excel attachments).

Draft Reports: A draft report shall be submitted to the Project Officer for each Validation Study phase (See **Section 4.2** and Addendum I). A Draft Final Report detailing the Contractor's involvement in all phases of the Validation Study shall be prepared by the Contractor, signed by the Study Director, and provided to the Project Officer. The submitted results shall accurately describe all methods used for generation and analysis of the data, provide a complete record of the preparation of test chemicals, and present any relevant data necessary for the assessment of the results (See Addendum I).

Final Report: The Draft Final Report shall be revised according to comments from the Project Officer and submitted as the Final Report (**See Section 4.2** and Addendum I).

9.0 RECORDS AND ARCHIVES

At the conclusion of the Contractor's participation in the distribution of chemicals for the Validation Study, the original raw and derived data, as well as copies of other raw data not exclusive to this Validation Study (instrument logs, calibration records, facility logs, etc.), shall be submitted to NIEHS/NICEATM (via the Project Officer) for storing and archiving according to the facility's SOP and in compliance with GLP Standards.

Originals of all raw and derived data, or copies where applicable, shall be stored and archived at NIEHS/NICEATM.

10.0 ALTERATIONS OF THE STATEMENT OF WORK

No changes in the Statement of Work shall be made without the consent of the Project Officer and Study Management Team. A Statement of Work Amendment detailing any change(s) and the basis for the change(s) shall be approved and prepared by the Study Director, and the amendment shall be signed and dated by the Study Director and the NIEHS representative. The amendment shall be retained with the original Statement of Work.

11.0 REFERENCES

Clonetics Normal Human Keratinocyte Systems Instructions for Use, AA-1000-4-Rev.03/00. (http://www.clonetics.com).

EPA Product Properties Test Guidelines. OPPTS 830.7840. 1998. Water Solubility: Column Elution Method; Shake Flask Method. United States Environmental Protection Agency. Prevention, Pesticides and Toxic Substances (7101). EPA 712-C-98-041. March 1998.

National Toxicological Program, September 2000, Attachment 2 revised. Specifications for the Conduct of Studies to Evaluate the Toxic and Carcinogenic Potential of Chemical, Biological and Physical Agents in Laboratory Animals for the National Toxicology Program (NTP).

NICEATM (The National Toxicology Program [NTP] Interagency Center for the Evaluation of Alternative Toxicological Methods). 2001. Test Method Protocol for the BALB/c 3T3 Neutral Red Uptake Cytotoxicity Test. A Test for Basal Cytotoxicity for an *In Vitro* Validation Study.

NICEATM (The National Toxicology Program [NTP] Interagency Center for the Evaluation of Alternative Toxicological Methods). 2001. Test Method Protocol for the Normal Human Keratinocyte [NHK] Neutral Red Uptake Cytotoxicity Test. A Test for Basal Cytotoxicity for an *In Vitro* Validation Study.

ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods). 2001. Guidance document on using *in vitro* data to estimate *in vivo* starting doses for acute toxicity NIH publication 01-4500. NIEHS, Research Triangle Park, North Carolina.

OECD (Organisation for Economic Co-operation and Development). 2001. Harmonised Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures as Endorsed by the 28th Joint Meeting of the Chemicals Committee and the Working Party on Chemicals in November 1998, Part 2, p. 21. OECD, Paris. http://www.oecd.org/ehs/class/HCL6htm.

12.0 APPROVAL OF STATEMENT OF WORK

Sponsor Representative	**Date**
Testing Facility Management	**Date**

ADDENDUM I

SUGGESTED REPORT FORMAT

TITLE PAGE

- **Study Title**
  - Draft Report 1: *Acquisition, Preparation, Solubility Testing, and Distribution of Test Chemicals: Phase I of the In Vitro Validation Study*
  - Draft Report 2: *Acquisition, Preparation, Solubility Testing, and Distribution of Test Chemicals: Phase II of the In Vitro Validation Study*
  - Draft Report 3: *Acquisition, Preparation, Solubility Testing, and Distribution of Test Chemicals: Phase III of the In Vitro Validation Study*
  - Draft/Final Report: *Acquisition, Preparation, Solubility Testing, and Distribution of Test Chemicals: Final Report for the In Vitro Validation Study*
- **Test Articles**
  - Draft Report 1: Identify the positive control chemical of Phase Ia and the three (3) test chemicals of Phase Ib
  - Draft Report 2: Identify the nine (9) test chemicals of Phase II
  - Draft Report 3: Identify the sixty (60) test chemicals of Phase III
  - Draft/Final Report: Identify all seventy-two (72) test chemicals and positive control of the *In Vitro* Validation Studies
- **Authors**
- **Study Completion Date**
- **Contract Facility**
- **Study Number/Identification**

SIGNATURE PAGE

- **Study Initiation Date:** Date Statement of Work was signed
- **Initiation Date of Laboratory Studies:** Actual laboratory start date
- **Study Completion Date:** Date report signed by Study Director
- **Sponsor Representative:**
  Ms. Molly Vallant – Project Officer
  The National Institute of Environmental Health Sciences (NIEHS)
- **Study Management Team Representatives**
  Judy Strickland, Ph.D. (Project Coordinator)
  Michael Paris (Assistant Project Coordinator)
- **Contractor Facility:** Name and address
- **Archive Location:** Name and address
- **Study Director:** Name and signature and date
- **Key Personnel:** Laboratory technicians, QA Director, Safety Officer
- **Facility Management:** Name
- **Scientific Advisor:** Name

ADDENDUM I (cont.)

DRAFT REPORT 1

Acquisition, Preparation, Solubility Testing, and Distribution of Test Chemicals: Phase I of the In Vitro Validation Study

- **Table of Contents**
- **Objectives :**The report shall provide specific objectives
- **Summary of the Findings:** Referenced to the raw data where appropriate; Include all information for the positive control (SLS) and the three (3) Phase Ib chemicals.
- **Narrative Description of the Solubility Studies:** Describe any problems that were encountered and how such problems were solved. Justifications for solvents used for each test chemical will be included in the description. Provide the information requested in **Section 7.1.1**. Deviations from the protocols, SOPs, and/or the Statement of Work shall be addressed in this section. Copies of appropriate sections of the Study Workbook shall be included with the report (as attachments). The draft report will include unaudited Study Workbook pages. The final report will include a copy of the audited Study Workbook with a statement (signed and dated by the Study Director) on the front of it stating that it is an exact copy of the original audited workbook.
- **Statement Signed by the Study Director:** Confirm that the solubility studies, acquisition, preparation, and distribution of the test chemicals were conducted in compliance with GLP (or indicating where the Study deviated from GLP). Confirm that the report fully and accurately reflects the raw data generated in the Study.
- **Other Information:** (All copies of documents will be noted as exact duplicates of the data.)
  - Information requested in **Section 7.1.2**
  - Deviations to the protocols, SOPs, and Statement of Work
  - Revisions/amendments to the protocols, SOPs, and Statement of Work

DRAFT REPORT 2

Acquisition, Preparation, Solubility Testing, and Distribution of Test Chemicals: Phase II of the In Vitro Validation Study

- **Table of Contents**
- **Objectives:** The report shall provide specific objectives
- **Summary of the Findings:** Referenced to the raw data where appropriate; Include all information for the nine (9) Phase II chemicals.
- **Narrative Description of the Solubility Studies:** Describe any problems that were encountered and how such problems were solved. Justifications for solvents used for each test chemical shall be included in the description. Provide the information requested in **Section 7.1.1**. Deviations from the protocols, SOPs, and/or the Statement of Work shall be addressed in this section. Copies of appropriate sections of the Study Workbook shall be included with the report (as attachments). The draft report will include unaudited Study Workbook pages. The final report will include a copy of the audited Study Workbook with a statement (signed and dated by the Study Director) on the front of it stating that it is an exact copy of the original audited workbook.
- **Statement Signed by the Study Director**: Confirm that the solubility studies, acquisition, preparation, and distribution of the test chemicals were conducted in compliance with GLP (or indicating where the Study deviated from GLP). Confirm that the report fully and accurately reflects the raw data generated in the Study.
- **Other Information:** (All copies of printouts, documents, and spreadsheets shall be noted as exact duplicates of the data.)
  - Information requested in **Section 7.1.2**
  - Deviations to the protocols, SOPs, and Statement of Work
  - Revisions/amendments to the protocols, SOPs, and Statement of Work

ADDENDUM I (cont.)

DRAFT REPORT 3

Acquisition, Preparation, Solubility Testing, and Distribution of Test Chemicals: Phase III of the In Vitro Validation Study

- **Table of Contents**
- **Objectives:** The report shall provide specific objectives
- **Summary of the Findings:** Referenced to the raw data where appropriate; Include all information for sixty (60) Phase III chemicals.
- **Narrative Description of the Solubility Studies:** Describe any problems that were encountered and how such problems were solved. Justifications for solvents used for each test chemical will be included in the description. Provide the information requested in **Section 7.1.1**. Deviations from the protocols, SOPs, and/or the Statement of Work shall be addressed in this section. Copies of appropriate sections of the Study Workbook shall be included with the report (as attachments). The draft report will include unaudited Study Workbook pages. The final report will include a copy of the audited Study Workbook with a statement (signed and dated by the Study Director) on the front of it stating that it is an exact copy of the original audited workbook.
- **Statement Signed by the Study Director:** Confirm that the solubility studies, acquisition, preparation, and distribution of the test chemicals were conducted in compliance with GLP (or indicating where the Study deviated from GLP). Confirm that the report fully and accurately reflects the raw data generated in the Study.
- **Other Information:** (All copies of printouts, documents, and spreadsheets shall be noted as exact duplicates of the data.)
  - Information requested in **Section 7.1.2**
  - Deviations to the protocols, SOPs, and Statement of Work
  - Revisions/amendments to the protocols, SOPs, and Statement of Work

DRAFT/FINAL REPORT

Acquisition, Preparation, Solubility Testing, and Distribution of Test Chemicals: Draft/Final Report for the In Vitro Validation Study

- **Table of Contents**
- **Objectives:** The draft/final report shall provide specific objectives
- **Summary of the Findings:** Referenced to the raw data where appropriate; Include all information for the seventy-two (72) test chemicals and the positive control (SLS).
- **Narrative Description of the Solubility Studies:** Describe any problems that were encountered and how such problems were solved. Justifications for solvents used for each test chemical shall be included in the description. Provide the information requested in **Section 10.1.1**. Deviations from the protocols, SOPs, and/or the Statement of Work shall be addressed in this section. Copies of appropriate sections of the Study Workbook shall be included with the report (as attachments). The draft report will include unaudited Study Workbook pages. The final report will include a copy of the audited Study Workbook with a statement (signed and dated by the Study Director) on the front of it stating that it is an exact copy of the original audited workbook.
- **Statement Signed by the Study Director:** Confirm that the acquisition, preparation, solubility studies, and distribution of the test chemicals were conducted in compliance with GLP (or indicating where the Study deviated from GLP). Confirm that the report fully and accurately reflects the raw data generated in the Study.
- **Quality Assurance Statement: (For Final Report only)**
  QA Statement identifying: 1) the phases and data inspected, 2) dates of inspection, and 3) dates findings were reported to the Study Director and Testing Facility management. The QA

Statement shall identify whether the methods and results described in the Final Report accurately reflect the raw data produced during the Study.

- **Other Information:** (All copies of printouts, documents, and spreadsheets shall be noted as exact duplicates of the data.)
  - Deviations to the protocols, SOPs, and Statement of Work
  - A list of all SOPs used by the laboratory (SOP title and laboratory identification code)
  - The Statement of Work

BIWEEKLY REPORTS

Contract Facility:

Chemicals Acquired:

Chemicals Tested for Solubility:

Results of Solubility Tests:

Chemicals Shipped to Testing Facilities:

Date of Shipping:

Problems Encountered/Resolutions:

Projected Shipping Schedule:

ADDENDUM II
SUGGESTED STANDARD TEST REPORTING TEMPLATE FOR STUDY WORKBOOK

[1]SOLUBILITY TESTING
Test Chemicals for the *In Vitro* Validation Study

Study No._____

Test Chemical_____ Test Chemical Code_____ CAS #_____

Physical Description_____ Liquid Density_____

Solubility Determined by_____
Date_____

Solvent	Amount of Test Chemical	Volume Added	Total Volume	pH and medium color	Vortex (V) Sonication (S) Heating-37°C (H)	Comments
Treatment Medium (3T3 NRU)		0.1ml				
		0.5ml				
		1.0ml				
Routine Culture Medium (NHK NRU)		0.1ml				
		0.5ml				
		1.0ml				
DMSO		0.1ml				
Ethanol		0.1ml				

Reference Color of Treatment Medium_____

Reference Color of Routine Culture Medium_____

Balance I.D._____
Treatment Medium and Routine Culture Medium: minimum concentration of 100mg/ml.
DMSO and Ethanol: minimum concentration of 1000mg/ml.

[1] Adaptation of Institute of In Vitro Sciences (IIVS) form – 350 [2/2002]

ADDENDUM III
TEST CHEMICALS FOR THE *IN VITRO* VALIDATION STUDY (ALPHABETICAL)

[NOTE: TESTING FACILITIES MUST NOT SEE THIS LIST OF CHEMICALS]

CHEMICAL	CAS NO.
1,1,1-Trichloroethane	71-55-6
2-Propanol	67-63-0
5-Aminosalicylic acid	89-57-6
Acetaminophen	103-90-2
Acetonitrile	75-05-8
Acetylsalicylic acid	50-78-2
~~**To be determined**~~[1]	
Aminopterin	54-62-6
Amitriptyline *HCl*[3]	~~50-48-6~~*549-18-8*[3]
Arsenic III trioxide	1327-53-3
Atropine sulfate *monohydrate*[3]	~~55-48-1, (~~*17108-73-5*~~)~~*73791-47-6*[3]
Boric aid	10043-35-3
Busulphan	55-98-1
Cadmium II chloride	10108-64-2
Caffeine	58-08-2
Carbamazepine	298-46-4
Carbon tetrachloride	56-23-5
Chloral hydrate	302-17-0
Chloramphenicol	56-75-7
Citric Acid	77-92-9
Colchicine	64-86-8
Cupric sulfate * 5 H2O	7758-99-8
Cycloheximide	66-81-9
Dibutylphthalate	84-74-2
Dichlorvos (DDVP)	62-73-7
Diethyl phthalate	84-66-2
Digoxin	20830-75-5
Dimethylformamide	68-12-2
Diquat	2764-72-9
Disulfoton	298-04-4
Endosulfan	115-29-7
Epinephrine bitartrate	51-42-3
Ethanol	64-17-5
Ethylene glycol	107-21-1
Fenpropathrin	39515-41-8
Gibberellic acid	77-06-5
Glutethimide	77-21-4
Glycerol	56-81-5
Haloperidol	52-86-8
Hexachlorophene	70-30-4
Lactic acid	50-21-5
Lindane	58-89-9

[1] Revised 5/23/02
[3] Revised 9/17/02

ADDENDUM III (CONT.)

CHEMICAL	CAS NO.
Lithium I ~~sulfate~~*carbonate*[3]	*554-13-2*~~10377-48-7~~[3]
Meprobamate	57-53-4
Mercury II chloride	7487-94-7
Methanol	67-56-1
Nicotine	54-11-5
Paraquat	1910-42-5, (*3765-78-4,57593-74-5,65982-50-5,136338-65-3,205105-68-6,247050-57-3*)[3]
Parathion	56-38-2
Phenobarbital	50-06-6
Phenol	108-95-2
Phenylthiourea	103-85-5
Physostigmine[1]	*57-47-6*[1]
Potassium cyanide	151-50-8
Potassium I chloride	7447-40-7
Procainamide *HCl*[3]	~~51-06-9~~*614-39-1*[3]
Propranolol HCl	318-98-9, (*3506-09-0, 146874-86-4*)[1]
Propylparaben	94-13-3
Sodium arsenite	7784-46-5
Sodium chloride	7647-14-5
Sodium dichromate dihydrate	7789-12-0
Sodium hypochlorite	8007-59-8, (*7681-52-9*)[3]
Sodium I fluoride	7681-49-4
Sodium oxalate	62-76-0
Sodium selenate~~*10 H20~~[1]	~~13413~~*13410*-01-0[1]
Strychnine	57-24-9
Thallium I sulfate	7446-18-6
Trichloroacetic acid	76-03-9
Triethylene melamine	51-18-3
Triphenyltin hydroxide	76-87-9
Valproic acid	99-66-1
Verapamil HCl	152-11-4
Xylene	1330-20-7

[3] Revised 9/17/02
[1] Revised 5/23/02

ADDENDUM IV
TEST CHEMICALS FOR THE *IN VITRO* VALIDATION STUDY BY STUDY PHASE

PHASE Ia	
Sodium laurel sulfate	151-21-3
PHASE Ib	
Arsenic III trioxide	1327-53-3
Ethylene glycol	107-21-1
Propranolol HCl	318-98-9, (*3506-09-0, 146874-86-4*)[1]
PHASE II	
Aminopterin	54-62-6
Chloramphenicol	56-75-7
Colchicine	64-86-8
Cupric sulfate * 5 H2O	7758-99-8
Lithium I ~~sulfate~~*carbonate*[3]	*554-13-2*~~10377-48-7~~[3]
Potassium I chloride	7447-40-7
2-Propanol	67-63-0
Sodium I fluoride	7681-49-4
Sodium selenate~~*10 H20~~[1]	~~13413~~*13410*-01-0[1]
PHASE III	
1,1,1-Trichloroethane	71-55-6
5-Aminosalicylic acid	89-57-6
Acetaminophen	103-90-2
Acetonitrile	75-05-8
Acetylsalicylic acid	50-78-2
~~**To be determined**~~[1]	
Amitriptyline *HCl*[3]	*549-18-8*~~50-48-6~~[3]
Atropine sulfate *monohydrate*[3]	*73791-47-6*~~55-48-1, (17108-73-5)~~[3]
Boric aid	10043-35-3
Busulphan	55-98-1
Cadmium II chloride	10108-64-2
Caffeine	58-08-2
Carbamazepine	298-46-4
Carbon tetrachloride	56-23-5
Chloral hydrate	302-17-0
Citric Acid	77-92-9
Cycloheximide	66-81-9
Dibutylphthalate	84-74-2
Dichlorvos (DDVP)	62-73-7
Diethyl phthalate	84-66-2
Digoxin	20830-75-5
Dimethylformamide	68-12-2
Diquat	2764-72-9
Disulfoton	298-04-4
Endosulfan	115-29-7
Epinephrine bitartrate	51-42-3

[3] Revised 9/17/02
[1] Revised 5/23/02

ADDENDUM IV (CONT.)

PHASE III (cont.)

Ethanol	64-17-5
Fenpropathrin	39515-41-8
Gibberellic acid	77-06-5
Glutethimide	77-21-4
Glycerol	56-81-5
Haloperidol	52-86-8
Hexachlorophene	70-30-4
Lactic acid	50-21-5
Lindane	58-89-9
Meprobamate	57-53-4
Mercury II chloride	7487-94-7
Methanol	67-56-1
Nicotine	54-11-5
Paraquat	1910-42-5, (*3765-78-4,57593-74-5,65982-50-5,136338-65-3,205105-68-6,247050-57-3*)[3]
Parathion	56-38-2
Phenobarbital	50-06-6
Phenol	108-95-2
Physostigmine[1]	*57-47-6*[1]
Phenylthiourea	103-85-5
Potassium cyanide	151-50-8
Procainamide *HCl*[3]	~~51-06-9~~*614-39-1*[3]
Propylparaben	94-13-3
Sodium arsenite	7784-46-5
Sodium chloride	7647-14-5
Sodium dichromate dihydrate	7789-12-0
Sodium hypochlorite	8007-59-8, (*7681-52-9*)[3]
Sodium oxalate	62-76-0
Strychnine	57-24-9
Thallium I sulfate	7446-18-6
Trichloroacetic acid	76-03-9
Triethylene melamine	51-18-3
Triphenyltin hydroxide	76-87-9
Valproic acid	99-66-1
Verapamil HCl	152-11-4
Xylene	1330-20-7

[1] Revised 5/23/02
[3] Revised 9/17/02

ADDENDUM V

ASSUMPTIONS FOR CALCULATION OF AMOUNT OF TEST MATERIAL NEEDED FOR EACH TESTING FACILITY

	Chemical Amount	Assumption
Phase I		
Test in 3 solvents	300 mg	Chemical must be tested in all 3 solvents
Test in 3 replicate assays	300	3 replicate assays must be performed
Repeat 3 times	300	3 replicate assays must be repeated 3 times
Phase I Amount/Testing Facility	900 mg	
x 3 Testing Facilities	2700	Assumes 3 labs participate in study
2 Archive samples (3 solubility + 3 assays)	1200	Archive samples use same amount of chemical as testing sample
Total Phase I Amount	3900 mg	
Phase II		
Test in 3 solvents	300 mg	Chemical must be tested in all 3 solvents
Test in 3 replicate assays	300	3 replicate assays must be performed
Repeat 2 times	200	2 replicate assays must be repeated 3 times
Phase II Amount/Testing Facility	800 mg	
x 3 Testing Facilities	2400	Assumes 3 labs participate in study
2 Archive samples (3 solubility + 3 assays)	1200	Archive samples use same amount of chemical as testing sample
Total Phase II Amount	3600 mg	
Phase III		
Test in 3 solvents	300 mg	Chemical must be tested in all 3 solvents
Test in 3 replicate assays	300	3 replicate assays must be performed
Phase III Amount/Testing Facility	600 mg	
x 3 Testing Facilities	1800	Assumes 3 labs participate in study
2 Archive samples (3 solubility + 3 assays)	1200	Archive samples use same amount of chemical as testing sample
Total Phase III Amount	3000 mg	

Specification of 4 g of chemical per Testing Facility in **Section 5.1.3** was chosen to allow a generous amount of error (in the direction of the Testing Facilities being provided with more chemical than necessary) in the calculations and assumptions made here.